

Normal Differentiation of Cultured Lens Cells after Inhibition of Gap Junction-Mediated Intercellular Communication

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The cells of the vertebrate lens are linked to each other by gap junctions, clusters of intercellular channels that mediate the direct transfer of low-molecular-weight substances between the cytosols of adjoining cells. Although gap junctions are detectable in the unspecialized epithelial cells that comprise the anterior face of the organ, both their number and size are greatly increased in the secondary fiber cells that differentiate from them at the lens equator. In other organs, gap junctions have been shown to play an important role in tissue development and differentiation. It has been proposed, although not experimentally tested, that this may be true in the lens as well. To investigate the function of gap junctions in the development of the lens, we have examined the effect of the gap junction blocker 18 β -glycyrrhetic acid (β GA) on the differentiation of primary cultures (both dissociated cell-derived monolayers and central epithelium explants) of embryonic chick lens epithelial cells. We found that β GA greatly reduced gap junction-mediated intercellular transfer of Lucifer yellow and biocytin throughout the 8-day culture period. β GA did not, however, affect the differentiation of these cells into MP28-expressing secondary fibers. Furthermore, inhibition of gap junctions had no apparent effect on either of the two other types of intercellular (adherens and tight) junctions present in the lens. We conclude that the high level of gap junctional intercellular communication characteristic of the lens equator *in vivo* is not required for secondary fiber formation as assayed in culture. Up-regulation of gap junctions is therefore likely to be a consequence rather than a cause of lens fiber differentiation and may primarily play a role in lens physiology. © 1998 Academic Press

INTRODUCTION

The lens is a solid cyst composed of only two cell types: a monolayer of epithelial cells that overlies its anterior face and a core of elongated, crystallin-rich fiber cells that is responsible for the refractive properties of the organ. These cells are physically and functionally linked to each other by gap junctions at their plasma membranes (Goodenough, 1992). Gap junctions are clusters of intercellular channels that link the cytosols of adjoining cells and thereby act as direct pathways for the cell-to-cell transfer of small (under ≈ 1 kDa) nutrients and signaling molecules. In vertebrates, gap junctions are composed of members of a family of structurally homologous integral membrane proteins

known as connexins which differ from each other with respect to their channel permeabilities, modes of regulation, and ability to interact with other connexin species (Bruzzone *et al.*, 1996; Goodenough *et al.*, 1996). Lens epithelial cells predominantly express connexin43 (Cx43), a widely distributed connexin that is believed to participate in cell–cell communication in a variety of embryonic and adult organs (Beyer *et al.*, 1987; Musil *et al.*, 1990a). Mature fiber cells lack Cx43 but instead express very high levels of two other connexin species. In the chick, these fiber-type connexins are referred to as Cx45.6 and Cx56 (Rup *et al.*, 1993; Jiang *et al.*, 1994); their orthologs in rodents are termed Cx50 and Cx46 (Paul *et al.*, 1991; White *et al.*, 1992). Because they lack biosynthetic or energy-producing organelles, mature fiber cells are uniquely dependent on the active transport and oxidative phosphorylation capacity of the anterior epithelial cells. Electrical impedance studies (Mathias and Rae, 1985) and analysis of intercellular coupling by diffusion of low-molecular-weight fluorescent dyes

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(Miller and Goodenough, 1986) or radiolabeled metabolites (Goodenough *et al.*, 1980) indicate that gap junctions join the lens cells into an ionic and metabolic syncytium. A role for gap junctions in lens transparency is supported by the recent finding that targeted disruption of Cx46 expression in mice results in the postnatal development of a nuclear cataract (Gong *et al.*, 1997).

Gap junctions are not specific to the lens but are instead found in almost all cell types in animals ranging from coelenterates to humans (Bennett *et al.*, 1991; Goodenough *et al.*, 1996). Two broad classes of functions have been ascribed to gap junctions. The first, similar to that described above for the lens, is to help maintain metabolic continuity within, and synchronize the function of, differentiated multicellular tissues. The second arises from numerous studies implicating gap junctions in various developmental processes, in which they have been proposed to mediate the intercellular transfer of morphogens and to help establish embryonic compartments (reviewed in Guthrie and Gilula, 1989; Warner, 1992; Lo, 1996). Reducing gap junctional intercellular communication with either function-blocking anti-connexin antibodies or pharmacological inhibitors of gap junction permeability disrupts early vertebrate embryogenesis (Warner *et al.*, 1984; Lee *et al.*, 1987) and limb patterning (Allen *et al.*, 1990). Studies utilizing dominant-negative connexin mutants or antisense mRNA constructs have supported a role for connexin-mediated intercellular communication in these processes (Bevilacqua *et al.*, 1989; Paul *et al.*, 1995). With regard to the differentiation of specific tissues, blockers of gap junction-mediated intercellular communication have been shown to reduce the expression of the muscle regulatory factors myogenin and MRF4 in skeletal myoblasts and the fusion of these cells into myotubes (Mege *et al.*, 1994; Proulx *et al.*, 1997a; Constantin *et al.*, 1997). Conversely, introduction of Cx43 into connexin-deficient rhabdomyosarcoma cells increases cell fusion and myosin expression concomitant with acquisition of gap junction-mediated intercellular coupling (Proulx *et al.*, 1997b). Restoration of gap junctional intercellular communication upon transfection with connexin-encoding cDNA has also been reported to increase the expression of tissue-specific genes (thyroglobulin and insulin, respectively) in cell lines derived from the thyroid (Statuto *et al.*, 1997) and from the pancreas (Vozzi *et al.*, 1995). In the mouse, elimination of Cx43 expression by targeted gene disruption results in lethal developmental malformations in the heart (Reaume *et al.*, 1995), whereas lack of connexin37-mediated oocyte-granulosa cell communication leads to female infertility by arresting oocyte development (Simon *et al.*, 1997).

Despite the presence of gap junctions throughout the lens and the importance of these structures in the development of other organs, the role of gap junctional intercellular communication in lens differentiation is not known. Although the time course and anatomical details differ between species, the general process by which the vertebrate lens develops is remarkably conserved between amphib-

ians, birds, and mammals, including humans (reviewed in Piatigorsky, 1981; Wride, 1996). Following induction, the embryonic ectoderm overlying the optic vesicle thickens to form the lens placode. The lens placode invaginates and eventually pinches off as the lens vesicle, a hollow sphere of epithelial cells. The cells at the posterior of the lens vesicle then differentiate into the primary fiber cells, which elongate to fill the lumen of the lens vesicle. In addition to an increase in cell volume, fiber cell differentiation is characterized by restructuring of the cell surface and cytosol, upregulation of fiber-specific proteins including various crystallins and the plasma membrane protein MP26 (known as MP28 in the chick), and eventual loss of intracellular organelles along with cessation of DNA synthesis and cell division. All subsequent growth of the lens (which continues throughout the life of the organism) is due to differentiation of epithelial cells into so-called "secondary" fiber cells, whose properties are similar to those of the primary fibers.

The process of secondary fiber formation begins with the progressive morphological and biochemical differentiation of a small population of epithelial cells near the equatorial axis of the lens. The equatorial region is also the site of the highest level of gap junctional intercellular coupling within the organ (Baldo and Mathias, 1992; Mathias *et al.*, 1997). The latter observation is likely to be due in part to the up-regulation of fiber-type connexin expression that occurs within this area (Berthoud *et al.*, 1994; Evans *et al.*, 1993; Gong *et al.*, 1997). In the chick, immunofluorescence and immunoelectron microscopy have demonstrated that the level of Cx43-containing gap junctions in equatorial epithelial cells also increases dramatically relative to the amount of Cx43 detected in the central epithelium at the anterior pole (Musil *et al.*, 1990a). Why synthesis and/or assembly of Cx43 should increase so substantially shortly before being shut off in mature fiber cells is unknown. Early studies of lens differentiation in culture have established the necessity of direct cell-cell contact in epithelial-to-fiber differentiation (Creighton *et al.*, 1976). Given the importance of gap junctions in other development processes, this could conceivably reflect a need for gap junctional intercellular communication in initiating and/or coordinating lens cell differentiation, a possibility raised previously by Menko as well as others (Menko *et al.*, 1987; Menko and Boettiger, 1988; Watanabe *et al.*, 1989). A potential role for gap junctions in lens development is supported by the observation that transformation of cultured chick lens epithelial cells with Rous sarcoma virus blocks both gap junctional intercellular communication and fiber cell differentiation (Menko and Boettiger, 1988). Furthermore, Watanabe *et al.* (1989) have reported that treatment of lens epithelial explants with anti-NCAM Fab fragments, while having no apparent effect on cell-cell contact per se, inhibits the formation of fiber-type gap junctions as well as cell elongation (a marker of initiation of fiber cell differentiation). Histological examination of the lenses of mice lacking Cx46 failed to reveal obvious defects in lens development

(Gong *et al.*, 1997). However, the fact that these mice still synthesize Cx43 and Cx50 (both of which are able to form functional gap junctions in the absence of Cx46) and continue to assemble the latter into lens fiber gap junctional plaques makes it very likely that a high level of cell-cell coupling is maintained at the cataract-free lens bow region. Thus, whether there is a causal relationship between gap junctional intercellular communication and lens differentiation has not been directly tested.

To address the role of gap junctions in epithelial-to-fiber maturation, we have used a derivative of glycyrrhetic acid (18 β -glycyrrhetic acid; β GGA) that has previously been demonstrated to potently inhibit gap junction-mediated intercellular dye and metabolite transfer in a variety of cell types (Davidson *et al.*, 1986; Guan *et al.*, 1996). Our results indicate that the extensive gap junctional intercellular communication characteristic of the equatorial region *in vivo* is not required for secondary fiber formation as assayed in two *in vitro* model systems of lens development. The primary function of gap junctions at the equator and throughout the lens may therefore be to support the metabolic and functional needs of the organ.

MATERIALS AND METHODS

Preparation of embryonic chick lens cultures. Dissociated cell-derived monolayer cultures were prepared using a modification of the procedure of Menko *et al.* (1984) as follows. Lenses were excised from E10 white leghorn chicken embryos and collected into TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5 mM D-glucose, 0.025 M Tris base, pH 7.4). Contaminating ciliary epithelium was removed by incubating the lenses in 0.08% trypsin in TD buffer at 37°C for 30 min and subsequent gentle trituration with a fire-polished Pasteur pipet. The cleaned lenses were then broken in M199 medium (Gibco BRL) supplemented with 10% fetal calf serum (Hyclone) and subjected to centrifugation at 1000 rpm for 10 min. The pelleted cells were resuspended in serum-free M199 medium and filtered through three layers of lens paper (A. H. Thomas) to remove capsule material and any cell clumps. The cells were then plated at near confluent density (1.8×10^5 cells/well) onto glass coverslips (Bellco) in a 96-well tissue culture plate in M199 plus OTS (25 μ g/ml ovotransferrin, 30 nM selenium) with penicillin G and streptomycin and maintained for up to 8 days at 37°C in a 5% CO₂ incubator. Prior to cell plating, each coverslip was treated overnight at 37°C with 0.5 mg/ml poly-D-lysine in 0.15 M borate buffer (pH 8.4), rinsed with distilled H₂O, and then coated with 0.03 mg/ml laminin in Earle's balanced salt solution (Gibco BRL) for 4–5 h at 37°C. Where indicated, cells were cultured in M199 supplemented with 15% fetal calf serum (Hyclone) instead of OTS.

Embryonic chick central epithelium explant cultures were prepared as described by Piatigorsky *et al.* (1972). E6 chick lenses were excised and placed in a 35-mm tissue culture dish containing M199 medium with the anterior epithelial cells closest to the dish. The posterior capsule was then punctured and the fiber mass was removed through the tear, leaving the epithelium intact. A scalpel was used to cut out a square of approximately 0.5 mm² of central epithelium, which was subsequently cultured in M199 medium

supplemented with 15% fetal calf serum, penicillin G, and streptomycin for up to 6 days at 37°C with 5% CO₂.

To inhibit gap junction-mediated intercellular communication, 18 β -glycyrrhetic acid (β GGA; Sigma) was added to the culture medium at the time of plating from a 100 mM stock in DMSO. Both dissociated cell-derived monolayers and central epithelium explants were fed every 2 days with fresh medium either with or without β GGA. Dissociated cell-derived monolayers treated with ≥ 40 μ M β GGA were cultured in the presence of 0.25% bovine serum albumin (Sigma) because concentrations of β GGA exceeding 10 μ M were toxic to lens cells in its absence.

Scrape-loading/dye transfer assay for gap junctional intercellular communication. Dissociated cell-derived chick lens monolayer cultures grown on laminin-coated glass coverslips in either the absence or presence of β GGA were transferred to a 35-mm tissue culture dish and their culture medium was saved. The transferred coverslips were then rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin and 1 mM CaCl₂ (HBC). The HBC was removed and 2.5 μ l of Dulbecco's phosphate-buffered saline (DPBS) containing 0.75% rhodamine dextran (Molecular Probes) with either 1% Lucifer yellow (Sigma) or 1% biocytin (Molecular Probes) was directly applied to the center of the glass coverslip, after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer. Cells were incubated in the dye mix for exactly 1 min and then quickly rinsed three times with HBC. After removal of the last rinse, the coverslip was encircled with a glass cloning cylinder to which the saved culture medium was added and the cells were incubated for 5 min (or, for Fig. 2, 15 min) at room temperature to allow dye transfer. Saved instead of fresh medium was used because of the rapid reversibility of the β GGA-induced block in gap junctional communication upon removal of the compound. The cells were then rinsed three times with PBS and immediately fixed for 30 min at room temperature with 2% paraformaldehyde/DPBS, pH 7.5. LY and rhodamine dextran were subsequently examined by fluorescence microscopy (Leitz DMR) using (respectively) fluorescein and rhodamine filter sets. For biocytin visualization, fixed cells were permeabilized for 15 min with DPBS containing 0.1% Triton X-100, 0.2% bovine serum albumin, and 5% normal goat serum followed by incubation with avidin-FITC (Molecular Probes) for 2 h at room temperature.

For embryonic chick epithelial explants, a modified scrape-loading/dye transfer assay was used because scratching the explant with a 27-gauge needle resulted in an unacceptable amount of tissue damage. A small punctate wound was created in the center of the explant using a micropipet containing either 1% Lucifer yellow/0.75% rhodamine dextran in DPBS or 1% biocytin/0.75% rhodamine dextran in DPBS under the control of a micromanipulator. Immediately after the cells were loaded with the dye mixture, the micropipet was removed and transfer was allowed to proceed for 2 min. The explants were maintained in their original culture medium (either with or without β GGA) throughout the procedure. The explant was then carefully rinsed three times with DPBS and fixed for 30 min with 2% paraformaldehyde in DPBS (pH 7.5). The dyes were visualized by fluorescence microscopy as described above for dissociated cell-derived monolayers.

Indirect immunofluorescence. Cells grown on laminin-coated glass coverslips or as epithelial explants were fixed in 2% paraformaldehyde in DPBS (pH 7.5) for 30 min at room temperature and rinsed in DPBS for 30 min. For Cx43 staining only, cells were then postfixed for 5 min with -20°C acetone followed by a 30-min rinse with DPBS. All cells were subsequently permeabilized in DPBS

containing 0.1% Triton X-100, 0.2% bovine serum albumin, and 5% normal goat serum (PTBN) for 30 min at room temperature followed by another rinse with DPBS for 30 min at room temperature. The primary antibody diluted in PTBN was applied to the fixed and permeabilized cells overnight at 4°C. After a 30-min rinse with PTBN at room temperature, the appropriate secondary antibody diluted in PTBN was added for 2 h at room temperature. The cells were given a final 30-min rinse in PTBN and then either mounted cell-side down on a microscope slide (in the case of coverslip-grown cells) or, for explants, topped with a coverslip using MOWIOL (Calbiochem) as a mounting medium.

The following previously characterized primary antibodies were used. Affinity-purified rabbit antibodies monospecific for Cx43 (AP7298) are described in Musil *et al.* (1990b). Rabbit antibodies directed against Cx45.6 or Cx56 were the kind gifts of Drs. J. Jiang and D. Goodenough, Harvard Medical School (Jiang *et al.*, 1995). Dr. Goodenough also provided polyclonal antibodies against chicken occludin (Chen *et al.*, 1997) and a mouse monoclonal antibody (R40.76) to ZO-1 (Anderson *et al.*, 1988). The anti-chicken MP28 polyclonal serum (No. 6182) and its preimmune control were generously provided by Dr. R. Johnson, University of Minnesota. This antiserum recognizes chick MP28 in whole lens lysates on Western blots and specifically stains differentiated fiber cells in frozen sections from E10 chick lens in a pattern indistinguishable from that obtained in duplicate sections with a monoclonal anti-MP28 antibody (Sas *et al.*, 1985). Rabbit anti-N-cadherin (C 3678) and anti- β -catenin antibodies (C 2206) were from Sigma. NCAM was detected with monoclonal antibody Mab 5E (Watanabe *et al.*, 1989).

[³⁵S]Methionine metabolic labeling and immunoprecipitation. Intact chick lenses dissected from E10 embryos, dissociated cell-derived monolayer cultures grown in 24-well laminin-coated tissue culture wells, or central epithelial explants were rinsed two times with Dulbecco's minimum essential medium (DMEM) without methionine and labeled with [³⁵S] methionine (EXPRE³⁵S³⁵S; New England Nuclear) in methionine-free DMEM at 37°C in either the absence or presence of the indicated concentration of β GA. Cultures grown in M199/OTS were labeled in the absence of serum; for all other cells, the labeling medium was supplemented with 5% dialyzed fetal calf serum. After the labeling period, the lenses or cultures were rinsed three times with 4°C TD buffer and then once with ice-cold lysis buffer (5 mM Tris base, 5 mM EDTA, 5 mM EGTA, 10 mM iodoacetamide, 2 mM PMSF, pH 8.0). The buffer was removed and the samples lysed by passage through an 18-gauge needle in 4°C lysis buffer supplemented with 0.6% SDS, 250 μ g/ml soybean trypsin inhibitor, and 200 μ M leupeptin. The lysates were then boiled for 3 min, followed by repeated passage through a 25-gauge needle to shear DNA released from lysed nuclei. Equal aliquots were removed for analysis of total cell lysates and the remainder diluted with 4 vol of immunoprecipitation buffer prior to immunoprecipitation with affinity-purified anti-Cx43 polyclonal antibodies (AP7298) as described in Musil *et al.* (1990a). The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels and quantitated on a PhosphorImager utilizing IPLab Gel software.

RESULTS

Differentiation of Primary Embryonic Chick Lens Cells in Dissociated Cell-Derived Monolayer Cultures

Although epithelial cells from the lens of many vertebrate species will take on fiber-like characteristics when

cultured, secondary fiber formation is most completely recapitulated by cells isolated from embryonic chick (Menko *et al.*, 1984, 1987; TenBroek *et al.*, 1994). We began our study by reproducing the culture conditions of Menko *et al.* (1984). E10 chick lenses were dissociated into single cells after removal of any nonlenticular contaminants and plated into tissue culture wells in M199 medium containing 15% fetal calf serum (M199/FCS). As expected, these cells proliferated to form a flattened epithelial monolayer. Beginning \approx 3–4 days of culture, discrete areas within these sheets differentiated into multilayered clusters of enlarged cells termed lentoids which increased in number and size over the remaining 4–5 days of the culture period (Fig. 1). Ultrastructural analysis by Menko *et al.* (1987) has revealed that the cells contained in such lentoids have acquired many of the defining characteristics of fiber cells in the intact embryonic chick lens, including increased cell volume, loss of intracellular organelles, and extensive formation of gap junctions. Thin-section electron micrographs of the cells closest to the substrate are indistinguishable from those obtained from the differentiated region of the embryonic lens, whereas the more peripheral cells closely resemble the elongating cells of the equatorial region (Menko *et al.*, 1987). Lentoids are also biochemically differentiated in that they accumulate high levels of δ -crystallin and of MP28 (also known as MIP) (Fig. 1H), an integral plasma membrane protein of as yet unclear function which is expressed *in vivo* only by differentiated primary and secondary fiber cells (Sas *et al.*, 1985; Yancey *et al.*, 1988).

We next defined the requirements for lentoid formation in dissociated cell-derived monolayers. We found that cells cultured in M199/OTS (M199 medium supplemented with 25 μ g/ml ovotransferrin and 30 nM selenium) in the absence of serum proliferated and differentiated into MP28-positive lentoids at a rate, and with a frequency, equivalent to serum-supplemented cells, although the lentoids tended to be somewhat smaller than in M199/FCS (Figs. 1A–1C and 1E–1G; compare 1C with 1D). Serum-independent differentiation was observed for cells plated onto laminin, collagen, uncoated tissue culture plastic, or Lab-tek glass chamber slides (see Fig. 5B), although under the latter two conditions the efficiency of initial cell attachment was reduced. The fact that lentoid production does not require the addition of exogenous and potentially nonphysiological growth factors to either the medium (in the form of fetal calf serum) or to the substrate (as a contaminant of extracellular matrix preparations) makes it likely that differentiation of these cultures replicates the *in vivo* process of secondary fiber formation as closely as possible. Unless otherwise noted, all experiments utilizing dissociated cell-derived cultures were conducted with cells grown in the absence of serum on coverslips coated with poly-D-lysine and laminin to ensure uniform plating density.

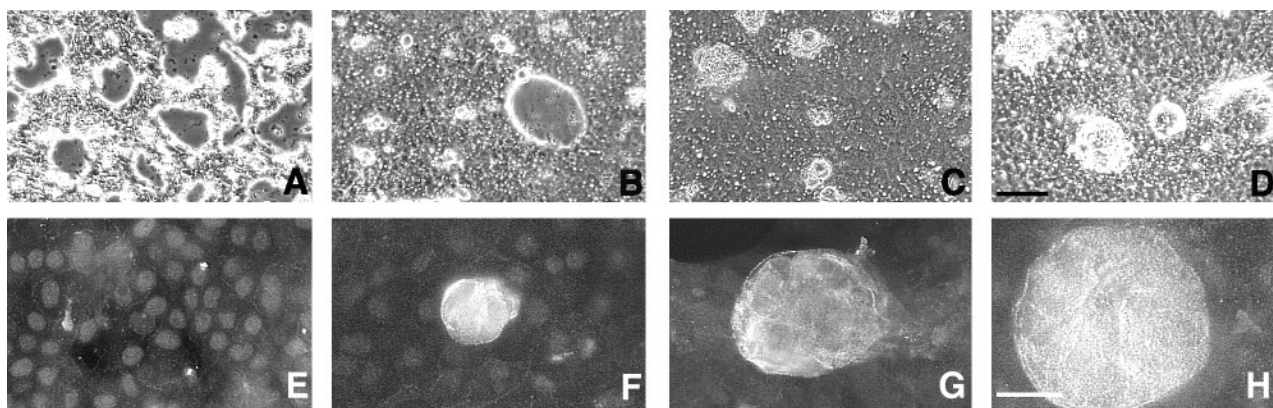


FIG. 1. Differentiation of dissociated cell-derived chick lens cultures. Primary cultures of embryonic chick lens cells were prepared from dissociated E10 lenses as described under Materials and Methods and plated in M199 with OTS (A–C, E–G) or M199 with 15% fetal calf serum (D, H). Phase-contrast micrographs (A–D) and immunofluorescence staining for the fiber cell-specific marker MP28 (E–H) of cells after 1 day (A, E), 3 days (B, F), or 8 days (C, D, G, H) in culture. The nuclear staining pattern evident in E was also obtained with preimmune serum (not shown); in contrast, staining of lentoid plasma membranes was specific to anti-MP28 immune serum. Bar: 100 μm (A–D); 20 μm (E–H).

Connexin Expression in Dissociated Cell-Derived Lens Cultures and the effect of βGA on Gap Junction-Mediated Intercellular Communication and Cx43 Processing

Immunofluorescence microscopy using antibodies specific for either Cx43, Cx45.6, or Cx56 demonstrated that the *in vivo* distribution of these connexins was recapitulated in our dissociated cell-derived cultures (see Figs. 6A–6C). As in the lenses of all avian and mammalian species examined to date, Cx43 was present at cell–cell interfaces throughout the epithelial monolayer during the entire culture period. In contrast, Cx45.6 and Cx56 were most concentrated in lentoids, consistent with their accumulation in fiber cells *in vivo*. These distributions were not, however, mutually exclusive. Epithelial monolayer cells also displayed readily detectable staining for Cx45.6 and Cx56, in keeping with the moderate expression of these connexins throughout the anterior epithelium of embryonic chick lenses *in vivo* (Jiang *et al.*, 1995). Furthermore, many cells within lentoids also stained brightly with the Cx43 antibody. This is likely due to the fact that the cells within a single lentoid mature asynchronously such that the cells nearest the surface of the structure are the least differentiated and are therefore the most similar to the elongating epithelial cells of the bow region *in vivo* (Menko *et al.*, 1984), in which Cx43 expression is even greater than in the central epithelium (Musil *et al.*, 1990a). Comparable connexin staining patterns and intensities were obtained with cultures maintained in the presence of fetal calf serum (data not shown).

As in intact lenses, the gap junctions expressed by cultured embryonic chick lens cells were functional (Figs. 2A and 2B). Gap junction-mediated intercellular coupling was

assessed using the scrape-loading/dye transfer assay, in which a mixture of rhodamine dextran (M_r 10 kDa) and the membrane impermeant, low-molecular-weight fluorescent dye Lucifer yellow (LY) is introduced into cultured cells by scraping the monolayer with a 27-gauge needle (el-Fouly *et al.*, 1987). The bulky dextran remains confined to the wounded cells, whereas LY is able to diffuse via gap junctions to adjacent cells distal to the scratch. As evaluated by the extent of transfer of LY to rhodamine dextran-negative cells, the monolayer lens cells were moderately well coupled throughout the culture period (Fig. 2 shows representative results from cells cultured for 3 days; other days were comparable). Minor local variation in the number of cells receiving LY could be due to uncoupling of cells during mitosis in these proliferating cultures (Stein *et al.*, 1992). In keeping with previous reports (Crow *et al.*, 1994), gap junction-mediated intercellular communication between monolayer epithelial cells was also readily detectable if LY was introduced into a single cell by microinjection (data not shown). The scrape-loading/dye transfer assay was routinely used since it allowed simultaneous monitoring of gap junctional intercellular communication within a larger population of cells.

Treatment of dissociated cell-derived chick lens cultures with 10 μM βGA in M199/OTS almost completely abolished the transfer of LY between epithelial monolayer cells, even if transfer of dye was allowed to proceed for 15 min instead of the standard 5 min (Fig. 2). As previously reported for fibroblast cell lines (Davidson *et al.*, 1986; Guan *et al.*, 1996), the effect of βGA on lens cell gap junction-mediated intercellular communication was dose-dependent ($\approx 50\%$ maximal effect at 5 μM βGA in M199/OTS), complete within 30 min, and reversible within 1 h of drug removal in

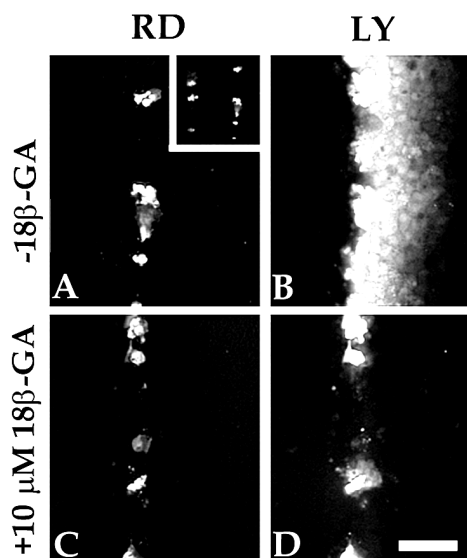


FIG. 2. β GA blocks gap junction-mediated intercellular transfer of Lucifer yellow in dissociated cell-derived chick lens monolayer cultures. Gap junctional intercellular communication was assessed in dissociated cell-derived chick lens monolayers cultured in M199/OTS in either the absence (A and B) or continuous presence (C and D) of 10 μ M 18 β -GA using a mixture of rhodamine dextran and Lucifer yellow as described under Material and Methods. The M_r 10 kDa rhodamine dextran (A and C) remains confined to the cells into which dye was directly introduced during the scrape/load process whereas Lucifer yellow (B and D) can be transferred to additional cells via open gap junctional channels during the 15-min transfer period. Representative results from day 3 of culture are shown. Each panel depicts a portion of the right half of the scrape/load wound (both sides of which are shown in the inset in A) which has been magnified to enhance the resolution of the individual cells. Bar: 100 μ m.

the presence of bovine serum albumin or serum (data not shown). Daily assessment of LY transfer in lens cells cultured in the continuous presence of 10 μ M β GA (with new β GA added only when the cell medium was changed every 48 h) revealed efficient inhibition of LY transfer between days 2 and 8 of culture relative to untreated control cells of the same age in 7/7 experiments. The persistence of the communication block throughout the 48-h period between medium changes demonstrated that β GA was not degraded or otherwise inactivated during this time. Although assessment of dye transfer on the first day of culture was less accurate due to the subconfluent state of the monolayer, β GA also substantially inhibited the number of rhodamine dextran-negative cells receiving LY at this time (data not shown). The fact that dissociated cell-derived chick lens cultures coexpress Cx43, Cx45.6, and Cx56 throughout the culture period indicates that β GA inhibits the function of not only Cx43 but also of the fiber-type connexins. This is an important point because gap junctional intercellular communication in vertebrate lens fibers

and in Cx56-expressing transfected tissue culture cells has been reported to be insensitive to certain other treatments (high CO_2 ; heptanol) that effectively block coupling mediated by other types of connexins (Miller and Goodenough, 1986; Rup *et al.*, 1993).

The limited transfer of LY and the small size of the monolayer epithelial cells made it difficult to appreciate the extent to which β GA inhibited gap junctional intercellular communication. We therefore repeated our analysis using biocytin as a tracer of gap junctional coupling (Fig. 3). Due

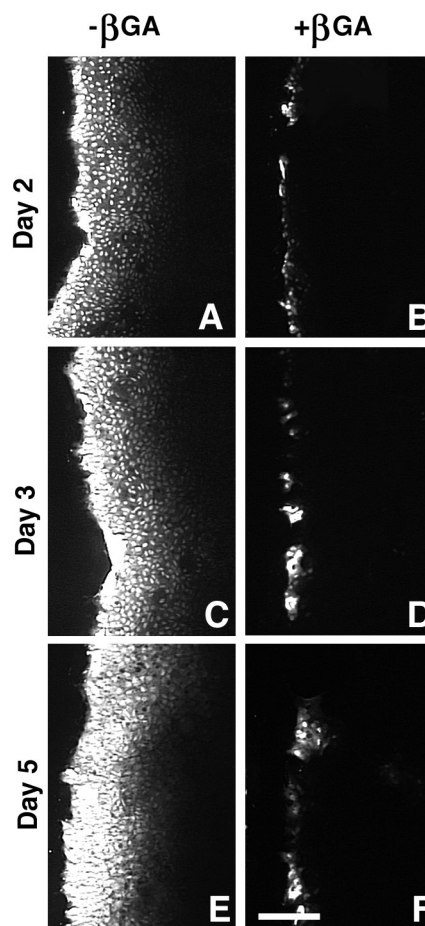


FIG. 3. β GA inhibits gap junction-mediated intercellular transfer of biocytin in dissociated cell-derived chick lens monolayer cultures. Gap junctional intercellular communication was assessed in dissociated cell-derived chick lens monolayers cultured for up to 8 days in M199/OTS in either the absence (A, C, E) or continuous presence (B, D, F) of 10 μ M 18 β -GA using a mixture of rhodamine dextran and biocytin as described under Material and Methods (5 min transfer period). Only the FITC-avidin staining for the biocytin is presented; rhodamine dextran (not shown) was confined to a single row of cells immediately bordering the wound (see Fig. 2). Representative results from day 2 (A, B), day 3 (C, D) and day 5 (E, F) of culture are presented. Only a portion of the right half of the wound is shown. Bar: 100 μ m.

in part to its lower molecular weight, ionic charge, and/or more sensitive method of detection (fluorochrome-labeled avidin instead of direct visualization of dye), biocytin has been shown in several cell types to transfer to more cells than simultaneously loaded LY (Teranishi and Negishi, 1994; Umino *et al.*, 1994). We found that this was also the case in untreated lens cells. Despite this increased sensitivity, intercellular transfer of biocytin was profoundly reduced by β GA throughout the culture period in 4/4 experiments (Figs. 3B, 3D, and 3F). Close inspection of scrape-loading/dye transfer assays did, however, reveal instances in which biocytin was detectable in rhodamine dextran-negative cells in the immediate vicinity of the wound. β GA therefore does not appear to totally abolish all gap junction-mediated intercellular communication in lens cells, in keeping with studies in which an even more sensitive assay of gap junction permeability (electrical coupling) detected residual intercellular communication in fibroblasts treated with other glycyrrhetic acid derivatives (Martin *et al.*, 1991; Goldberg *et al.*, 1996; see Discussion).

In gap junctional communication-competent cell types including embryonic chick lens epithelial cells, Cx43 is synthesized as an ≈ 42 -kDa species that undergoes phosphorylation to two slower-migrating forms, referred to as Cx43-P₁ and P₂, after transport to the cell surface (Musil *et al.*, 1990a,b). It is the P₂ species that is most closely correlated with functional gap junctional plaque formation (Musil and Goodenough, 1991). Guan *et al.* (1996) have reported that when intercellular communication is blocked in WB-F344 rat liver epithelial cells with 40 μ M β GA, Cx43 is no longer detectable in the P₂ form and that this species reappears only when cell coupling is restored by removal of the drug. We found that 40 μ M β GA similarly abolished phosphorylation of newly synthesized Cx43 to the P₂ form in whole embryonic chick lenses metabolically labeled with [³⁵S]methionine (Fig. 4A; compare lanes 1 and 2). Although the P₁ and P₂ species were not well resolved, 100 μ M β GA greatly reduced the amount of phosphorylated (>42-kDa) Cx43 recovered from dissociated cell-derived cultures (Fig. 4A, compare lanes 3 and 4). Thus, biochemical as well as functional assays indicated that ≥ 40 μ M β GA affects Cx43 in the lens in a manner similar to that described in other cell types. However, the phosphorylation pattern of newly synthesized Cx43 was unaltered by lower levels of β GA (10 μ M) that nonetheless maximally inhibited gap junction-mediated intercellular communication (Fig. 4A; compare lanes 5 and 6). Reduced phosphorylation of Cx43 is therefore not obligatorily linked to inhibition of gap junctional permeability and may instead reflect a separate effect of higher concentrations of β GA.

Differentiation of Dissociated Cell-Derived Chick Lens Cultures in the Presence of β GA

The effect of chronic inhibition of gap junctional intercellular communication was evaluated in dissociated embryonic chick lens cells plated and cultured in the continu-

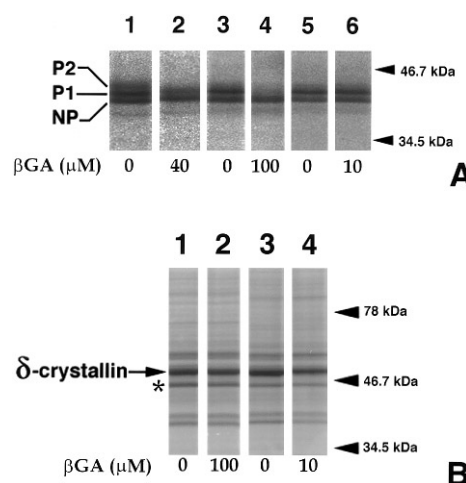


FIG. 4. Effect of β GA on metabolic labeling of connexin43 and δ -crystallin in embryonic chick lenses and dissociated cell-derived cultures. (A) Dissociated cell-derived chick lens monolayer cultures were propagated in M199/OTS for 8 days with 0 (lanes 3 and 5), 100 μ M (lane 4), or 10 μ M (lane 6) 18 β -GA and then metabolically labeled for 2 h with [³⁵S]methionine (0.13 mCi/ml) in the presence of the same concentration of β GA. For comparison, freshly isolated intact E10 chick lenses were labeled with [³⁵S]methionine for 2 h in either the absence (lane 1) or presence (lane 2) of 40 μ M 18 β -GA. The labeled cells were solubilized in SDS and the resulting lysates were immunoprecipitated with affinity-purified rabbit anti-Cx43 antibodies as described under Materials and Methods. The nonphosphorylated (NP) and two phosphorylated (P₁ and P₂) forms of Cx43 were visualized after SDS-PAGE using a PhosphorImager. B shows equal amounts of [³⁵S]methionine-labeled total cell lysates (2-h labeling period) from dissociated cell-derived chick lens monolayers cultured for 8 days with 0 (lanes 1 and 3), 100 μ M (lane 2), or 10 μ M (lane 4) 18 β -GA. Coomassie blue staining of whole lens lysates confirmed the identity of the δ -crystallin band (not shown). When normalized to actin (indicated by the asterisk), synthesis of δ -crystallin in cultures treated with 100 μ M β GA was 1 \times , and with 10 μ M β GA 1.3 \times , of their respective untreated controls. In both A and B, cells were cultured with 100 μ M 18 β -GA in the presence of 0.25% bovine serum albumin since concentrations of β GA >10 μ M were toxic to lens cells in its absence. Molecular weight markers are indicated on the right.

ous presence of 10 μ M β GA in M199/OTS (Fig. 5). β GA did not visibly affect the initial attachment or spreading of lens cells onto the substratum nor significantly change the size or the number of cells per well on days 1–3 of culture (data not shown; inefficient trypsinization of lentoids precluded accurate cell counts at later developmental stages). Most importantly, despite the ongoing block of gap junction-mediated intercellular communication, β GA-treated cultures continued to differentiate into MP28-expressing lentoids at a rate equal to untreated cells throughout the 8-day culture period (Fig. 5 shows data from a late time point). Although the onset and extent of lentoid formation showed

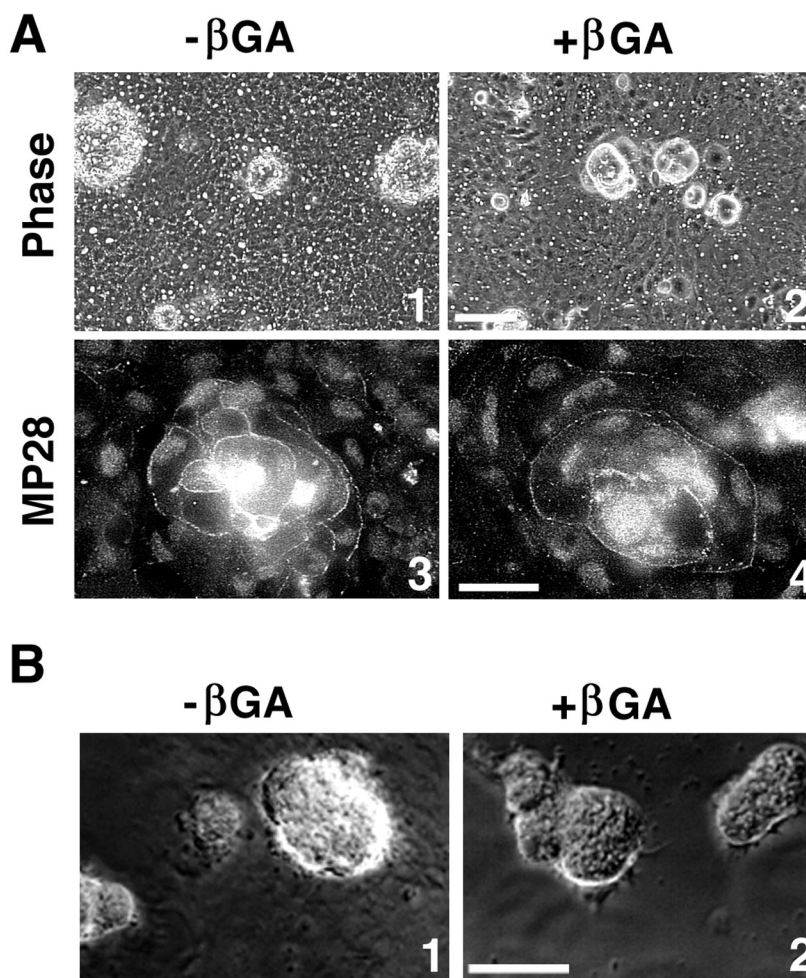


FIG. 5. Inhibition of gap junctional communication with β GA does not affect the differentiation of dissociated cell-derived chick lens monolayer cultures. Primary embryonic chick lens cells were cultured in M199/OTS for 8 days either on poly-D-lysine/laminin-coated coverslips (A, 1–4) or on uncoated glass Labtek chamber slides (B, 1 and 2) in either the absence (A1, A3; B1) or continuous presence (A2, A4; B2) of 10 μ M 18 β -GA. (A, 1 and 2, and B) Phase-contrast micrographs. (A, 3 and 4) Lentoids were visualized with a polyclonal antiserum against chicken MP28 followed by rhodamine-conjugated goat anti-rabbit IgG. Bar: 100 μ m (A1, A2); 20 μ m (A3, A4); 100 μ m (B1, B2).

slight culture-to-culture variability, in 21/21 experiments the course of lentoid formation was not noticeably affected by the presence of β GA. The [35 S]methionine-labeled (Fig. 4B, lane 4) and Coomassie blue-stained (not shown) profiles of total proteins synthesized by treated cultures were very similar to those of control cells and included high levels of δ -crystallin, a major component of differentiated embryonic chick fiber cells (Piatigorsky, 1981). Secondary fiber differentiation also proceeded normally in cells cultured with β GA in the presence of 0.25% bovine serum albumin or 15% fetal calf serum, for which a 10-fold higher concentration of β GA was required to achieve a comparable inhibition of gap junctional communication due to binding of β GA to albumin (data not shown). As previously shown for human lens epithelial cells (Arita *et al.*, 1990), embryonic

chick lens cells do not spread efficiently when plated onto less haptotactic substrates and instead show accelerated differentiation into lentoid bodies. Even under these extreme conditions, β GA did not inhibit lentoid formation (Fig. 5B).

With regard to the expression of connexins, we found that Cx43 in β GA-treated cultures was still localized to what appears at the level of resolution of immunofluorescence light microscopy to be cell-cell interfaces (Fig. 6D). Likewise, the distribution of the fiber-type connexins was not notably altered by exposure to β GA and remained detectable throughout the monolayer, with the highest concentration in the lentoids (Figs. 6E and 6F). A similar distribution of connexins was observed in cells cultured with 100 μ M β GA in the presence of 0.25% bovine serum albumin.

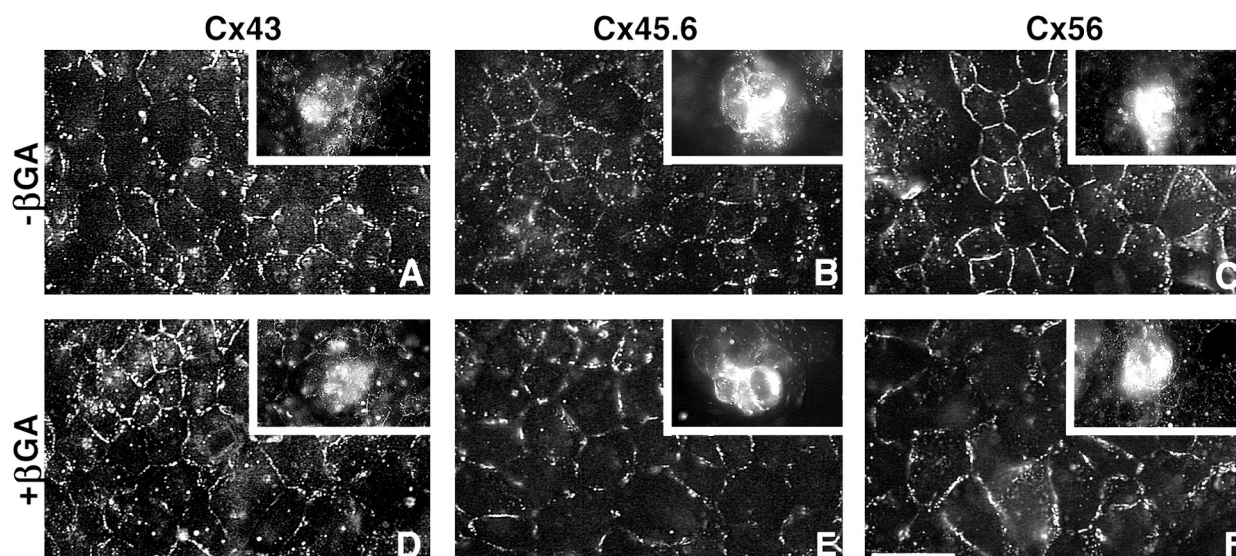


FIG. 6. Immunofluorescence localization of connexins in dissociated cell-derived chick lens monolayer cultures is not affected by β GA. Cells cultured in M199/OTS for 8 days in either the absence (A, B, C) or continuous presence (D, E, F) of levels of 18β -GA ($10\ \mu\text{M}$) that inhibit gap junction-mediated intercellular dye transfer were stained with antibodies specific for Cx43 (A, D), Cx45.6 (B, E), or Cx56 (C, F). Bar: $20\ \mu\text{m}$. As visualized by indirect immunofluorescence microscopy, all three connexins were concentrated at cell-cell interfaces throughout the monolayer of polygonal epithelial cells and were also detectable in differentiating multilayered lentoids (insets), in which the staining of the fiber connexins is so intense that it no longer outlines individual cells (B, C, E, and F). No staining was detected in cultures incubated with preimmune or normal rabbit serum (data not shown).

In untreated cultures, the staining pattern for each of the three connexins at cell-cell interfaces is often more continuous than (as is typical in most other cell types) concentrated in discrete puncta (especially prominent in Fig. 6C). It is perhaps for this reason that we do not observe by immunofluorescence microscopy an apparent disassembly of discrete gap junctional plaques in the presence of β GA as has been reported by Guan *et al.* (1996) in WB-F344 rat liver epithelial cells. Alternatively, the fact that Goldberg *et al.* (1996) could detect changes in gap junction morphology by freeze-fracture electron microscopy but not by light microscopy after exposure of C6 glioma cells to uncoupling levels of the related compound 18α -carbenoxolone suggests that the extent of morphological perturbation of gap junction structure induced by glycyrrhetic acid derivatives may vary between cell types.

Effect of Inhibition of Gap Junction-Mediated Intercellular Communication on Other Cell-Cell Junctions

As postulated by the "precedence hypothesis" (Edelman, 1988), several studies have indicated that cadherin-mediated intercellular adhesion is necessary not only for formation of adherens junctions but also for the assembly of other cell-cell specializations, including gap junctions, tight junctions, and desmosomes in various cell types (Gumbiner *et al.*, 1988; Musil *et al.*, 1990b). Given that

function-blocking anti-N-cadherin antibodies have been reported to inhibit the establishment of gap junctional intercellular communication between cultured embryonic chick lentoids (Frenzel and Johnson, 1996), this is likely to be true in the lens as well. Evidence is accumulating that cell-cell adhesion may also be reciprocally influenced by gap junction-mediated intercellular communication, in that inhibition of gap junctions with anti-connexin antibodies or via expression of a dominant-negative connexin mutant leads to disruption of cell-cell contact in (respectively) tissue culture cells (Meyer *et al.*, 1992) and early *Xenopus* embryos (Paul *et al.*, 1995). We therefore used β GA to investigate the role of gap junctional intercellular communication in the formation of other lens cell-cell junctions.

Adherens junctions. Studies by Geiger and colleagues have established that chick lens epithelial cells both *in vivo* and in culture assemble N-cadherin (A-CAM) into adherens junctions and that this activity is required for close cell-cell apposition (Duband *et al.*, 1988; Volk and Geiger, 1986). As assessed by immunofluorescence microscopy using an antibody raised against chick N-cadherin, β GA treatment has no discernible effect on the expression or distribution of this molecule at cell-cell interfaces (Figs. 7A and 7C). Strong cell-cell adhesion is thought to require cadherins to be linked to the actin-based cytoskeleton via catenins, an interaction commonly assessed by resistance to solubilization with the nonionic detergent NP-40 (Hirano *et al.*, 1987;

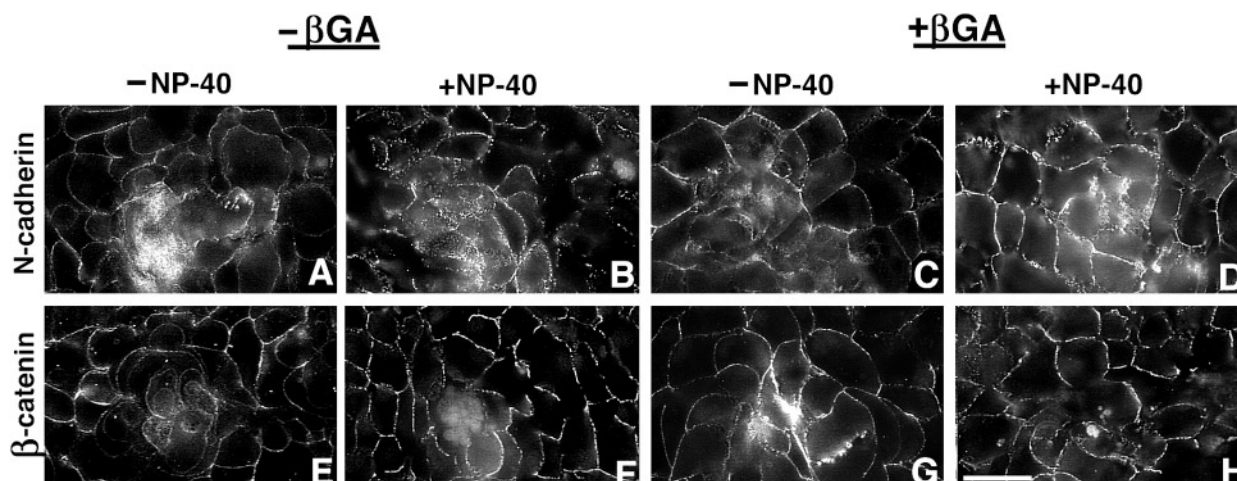


FIG. 7. Expression and detergent extractability of adherens junction proteins in chick lens cell monolayers $\pm\beta$ GA. Dissociated cell-derived chick lens monolayer cultures were propagated in M199/OTS for 8 days in either the absence (A, B, E, F) or presence (C, D, G, H) of 10 μ M 18 β -GA. The cultures were then incubated for 5 min at 4°C in calcium-containing PBS either with (B, F, D, H) or without (A, E, C, G) 5% NP-40 detergent. After four gentle rinses with Ca^{2+} PBS, the cells were fixed and stained for either N-cadherin (A–D) or β -catenin (E–H) followed by rhodamine-conjugated goat anti-rabbit IgG. Bar: 20 μ m.

Kreft *et al.*, 1997). In both control and β GA-treated lens cells, the majority of immunofluorescently detectable N-cadherin and β -catenin was not extractable with 5% NP-40 (Fig. 7). Control experiments in which the transport blocker brefeldin A was used to accumulate intracellular (and therefore NP-40 soluble) pools of cadherin confirmed that NP-40 efficiently extracted N-cadherin not associated with the cytoskeleton (data not shown). Taken together with the observation that β GA-treated lens cells maintain apparently normal close appositions, we conclude that inhibition of gap junction-mediated intercellular communication does not deleteriously affect cadherin localization or function. As assessed by rhodamine-phalloidin visualization of stress fibers and antivinculin staining, β GA also did not noticeably affect actin-dependent cell-substrate interactions (data not shown).

Tight junctions. Tight junctions have been identified by freeze–fracture electron microscopy in the chick lens (Goodenough *et al.*, 1980). These structures also appear to be assembled by dissociated cell-derived chick lens cultures as assessed by the immunofluorescence localization of the tight junction-specific integral plasma membrane protein occludin and the peripheral membrane protein ZO-1 (Figs. 8A and 8C). Both proteins were detected in the “chicken wire” cell interface staining pattern characteristic of tight junctions in epithelial monolayers (Wong and Gumbiner, 1997). Neither the distribution nor amount of these proteins was detectably altered in β GA-treated cells (Figs. 8B and 8D). The apparent lack of effect of β GA on lens tight junctions is in keeping with a recent study by De Sousa *et al.* (1997) in which epithelial integrity was maintained in Cx43-null early mouse embryos despite their severely reduced capacity to mediate intercellular dye transfer.

NCAM. In addition to cadherins, lens epithelial and cortical fiber cells express the Ca^{2+} -independent cell–cell adhesion molecule NCAM. Anti-NCAM antibodies have been reported to inhibit the formation of fiber-type gap junctions in embryonic chick lens explants (Watanabe *et al.*, 1989), suggesting that there may be a functional relationship between NCAM and gap junctions. However, β GA inhibition of gap junctional intercellular communication had no apparent effect on NCAM expression or localization as assessed by immunofluorescence (Figs. 8E and 8F).

Differentiation of Central Epithelium Explants in the Presence or Absence of β GA

Studies in rodent lenses have demonstrated regional differences in the inherent capacity of cells derived from different areas of the lens to develop into fibers, with the central epithelium being the most dependent on exogenous growth factors for differentiation (Richardson *et al.*, 1992). The finding that our dissociated cell-derived cultures form lentoids even in the absence of added growth factors suggests that many of the cells may have originated from the more peripheral (bow) regions of the lens. It is therefore possible that a requirement for gap junction-mediated intercellular communication, like that for exogenous growth factors, might be greater for differentiation of central epithelial cells than for more peripheral populations and that such a distinction might not be detectable in the heterogeneous mixture of cells cultured from dissociated lenses. To address this issue, we prepared lens explants in which the intact central epithelial monolayer is cultured capsule-side down after manual extraction of the fiber cell mass (Philpott and Coulombre, 1968; Piatigorsky *et al.*, 1973; Wa-

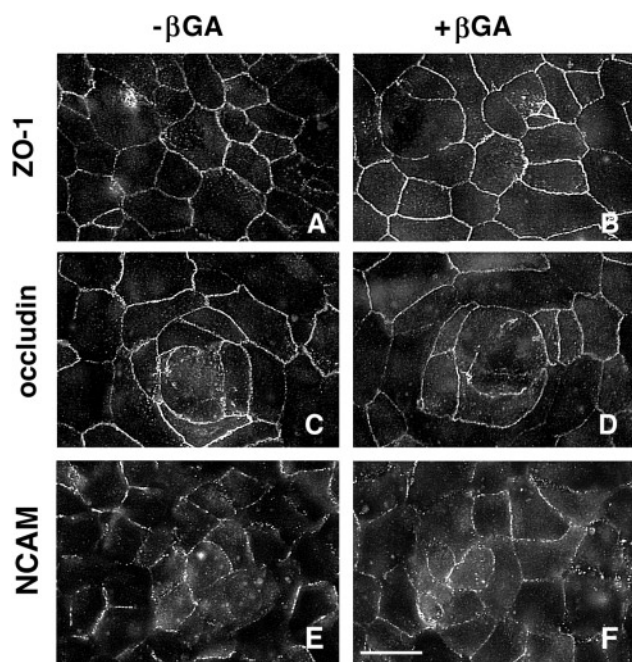


FIG. 8. Localization of tight junction components and NCAM in cultured lens cells is not altered by inhibition of gap junctions. ZO-1 (A, B), occludin (C, D), and NCAM (E, F) were visualized by indirect immunofluorescence microscopy in chick lens cells cultured for 8 days in either the absence (A, C, E) or continuous presence (B, D, F) of 10 μ M 18 β -GA. Bar: 20 μ m.

tanabe *et al.*, 1989). In addition to removing the peripheral epithelial cells, this well-established system (unlike cultures derived from dissociated cells) preserves cell–cell and cell–extracellular matrix interactions established *in vivo*, either of which could conceivably influence the differentiation process.

As expected from the literature (Piatigorsky, 1973), central epithelium explants were viable but remained as an undifferentiated, flat epithelial sheet when maintained in the absence of serum or another source of exogenous growth factors. If cultured for more than 3 days in the presence of 15% fetal calf serum, however, the cells, especially those at the periphery of the explant, became elongated. Although this thickening made it difficult to resolve individual cells by conventional light microscopy, it is clear that the elongated cells expressed high levels of the fiber cell-specific marker MP28 (Figs. 9B and 9E). Moreover, as reported by Piatigorsky (Piatigorsky *et al.*, 1973) and others, such explants increased their synthesis of δ -crystallin, another protein whose expression is upregulated during fiber formation (Fig. 11). As assessed by a modified scrape-loading/dye transfer assay, the cells in explants cultured for 1 day were moderately well coupled by gap junctions (Figs. 10A, panels 1–3, and 10B, panels 1–3). Treatment of explants with 100 μ M β GA in the presence of 15% fetal calf

serum reduced the amount of intercellular transfer of Lucifer yellow (Fig. 10A, panels 4–6) or biocytin (Fig. 10B, 4–6) to an extent comparable to that observed in β GA-treated dissociated cell-derived cultures (compare Fig. 10 with Figs. 2 and 3) in 5/5 experiments. Neither the morphological (Fig. 9) nor biochemical (as assessed by expression of MP28 and δ -crystallin; Figs. 9 and 11) differentiation of central epithelium explants into fiber-like cells was detectably inhibited by the continuous inhibition of gap junctional intercellular communication by 100 μ M β GA in 8/8 separate experiments.

DISCUSSION

The abundance and likely functional significance of gap junctions in the lens have made this organ the focus of extensive study of gap junction structure, biochemistry, and physiology (Goodenough, 1992; Zampighi *et al.*, 1992). Despite this level of investigation and precedence in other tissues, the role of gap junctions in the development of the lens is unknown. Given the lack of a specific blocker of gap junction function that is suitable for long-term use in whole animals, there are currently two approaches to this question. The first is to use targeted gene disruption to abolish connexin expression. Because there are three known functional connexins in the vertebrate lens, this would require the generation of a “triple knock-out” in which extralenticular developmental defects might complicate the interpretation of any phenotype. It is also possible that additional, as yet uncharacterized connexin species may be expressed in the lens, necessitating the direct functional evaluation of intercellular communication in the lenses of such mice. Precedence for unanticipated connexin species functionally coupling cells in the absence of the predominant connexin is provided by the observation that targeted gene disruption of Cx43 reduces, but does not eliminate, gap junctional communication in preimplantation mouse embryos due to the presence of connexin45 (De Sousa *et al.*, 1997). Furthermore, the fact that Cx43 expression begins at the initial stages of lens development and that of the fiber-type connexins shortly thereafter (Evans *et al.*, 1993; Jiang *et al.*, 1995) raises the possibility that any observed abnormality in secondary fiber formation could be an indirect consequence of defects in a preceding step in lens development or be due to general cellular dysfunction arising from chronic disruption of gap junction-dependent lens metabolism. For these reasons, we believe that an *in vitro* approach, in which the level of gap junctional intercellular communication can be acutely manipulated and is unlikely to dictate nutrient access, provides valuable insights into the role of gap junctions in lens development that are not obtainable *in vivo*.

For as yet unknown reasons, *in vitro* epithelial-to-fiber maturation is most complete in chick cells, with the most differentiated cells obtained in culture indistinguishable on the ultrastructural level from secondary fiber cells in whole

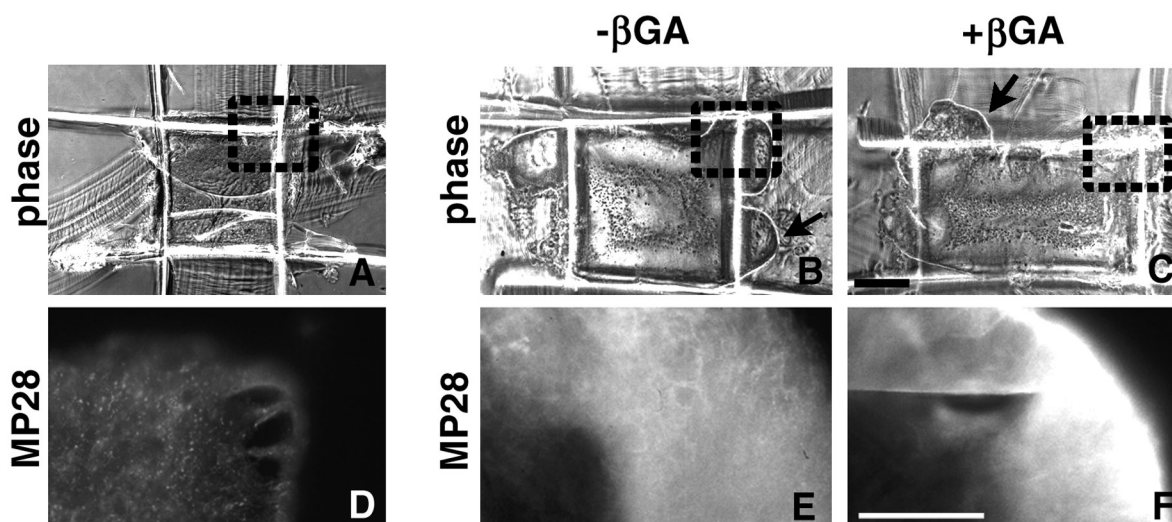


FIG. 9. Inhibition of gap junctional communication with β GA does not affect the differentiation of embryonic chick lens central epithelium explants. Explants were prepared from E6 lenses as described under Materials and Methods. Immediately after dissection, the explants consisted of a monolayer of flat epithelial cells completely contained within the central square of the grid pattern caused by gouging of the tissue culture plastic during explant preparation (A). When cultured in M199/15% fetal calf serum for 6 days in either the absence (B and E) or the continuous presence (C and F) of gap junction-blocking levels of 18β -GA ($100 \mu\text{M}$; see Fig. 10), these cells differentiated into elongated, fiber-like cells that extend over the original boundaries of the explant (most evident in the region indicated by the arrow in B and C). These peripheral regions expressed very high levels of the fiber-specific marker MP28 as assessed by immunofluorescence (E and F); the much lower, patchy staining for MP28 observed in day 0 explants (D) most likely originates from fragments of fiber cell membranes that remain adhered to the epithelial monolayer during explant preparation (see Fig. 3B in Jiang *et al.*, 1995). The area boxed in A, B, and C denotes the perimeter of the field shown in D, E, and F, respectively. Bar: $100 \mu\text{m}$ (A, B, C); $20 \mu\text{m}$ (D, E, F).

lens cortex (Menko *et al.*, 1984, 1987). We therefore chose to use primary embryonic chick lens cells for our study. Given the morphological, functional, and biochemical similarities in the development of the lens between birds and mammals (Piatigorsky, 1981), it is very likely that our findings will extend to higher vertebrates as well.

We examined the role of gap junctions in lens development using β GA, one of several glycyrrhetic acid derivatives that have been used to block gap junction permeability in cultured cells (Davidson, 1986; Goldberg *et al.*, 1996; Guan *et al.*, 1996). β GA has been shown (as were two other unrelated inhibitors of gap junction function) to inhibit cell-cell coupling and myogenesis of cultured rat L6 myoblasts and of primary embryonic chick myoblasts, illustrating the utility of this compound for addressing the role of gap junctions in differentiation processes *in vitro* (Proulx *et al.*, 1997a; Mege *et al.*, 1994). As documented for other cell types, β GA rapidly (within 30 min) and continuously (if replaced every 2 days) suppressed gap junction-mediated intercellular transfer of Lucifer yellow and biocytin in cultured embryonic chick lens cells without noticeable toxic effects. We found that in the absence of serum, maximal inhibition of gap junctional communication in dissociated cell-derived lens cultures was achieved using 4–10 times lower β GA concentrations than those generally used in its presence, reducing the possibility of nonspecific

effects of the drug. Unlike β GA, other reported gap junction blockers (heptanol, 18α -carbenoxolone, oleamide, anandamide) eventually killed lens cells under our culture conditions when used at concentrations required to inhibit junctional permeability (data not shown). We found that inhibition of gap junction-mediated intercellular communication by β GA did not appreciably disrupt either the plating or (as assessed by the formation of MP28-expressing lentoids) the epithelial-to-secondary fiber differentiation of dissociated cell-derived chick primary lens cultures throughout the 8-day culture period. Furthermore, β GA did not alter the expression of adherens or tight junctions as detected by light microscopy.

Interpretation of these results requires consideration of two properties of glycyrrhetic acid derivatives. First, β GA (like all other known gap junction uncouplers) is not completely specific for connexins and has been reported to affect certain other proteins as well (Davidson *et al.*, 1986). Although this could have been problematic had β GA been shown to perturb fiber differentiation, it seems unlikely that a gap junction-independent activity of glycyrrhetic acid somehow compensates for a requirement for gap junction-mediated intercellular communication in fiber differentiation. Second, as judged from the very low but detectable intercellular transfer of biocytin in β GA-treated lens cells, the inhibition of gap junctional intercellular

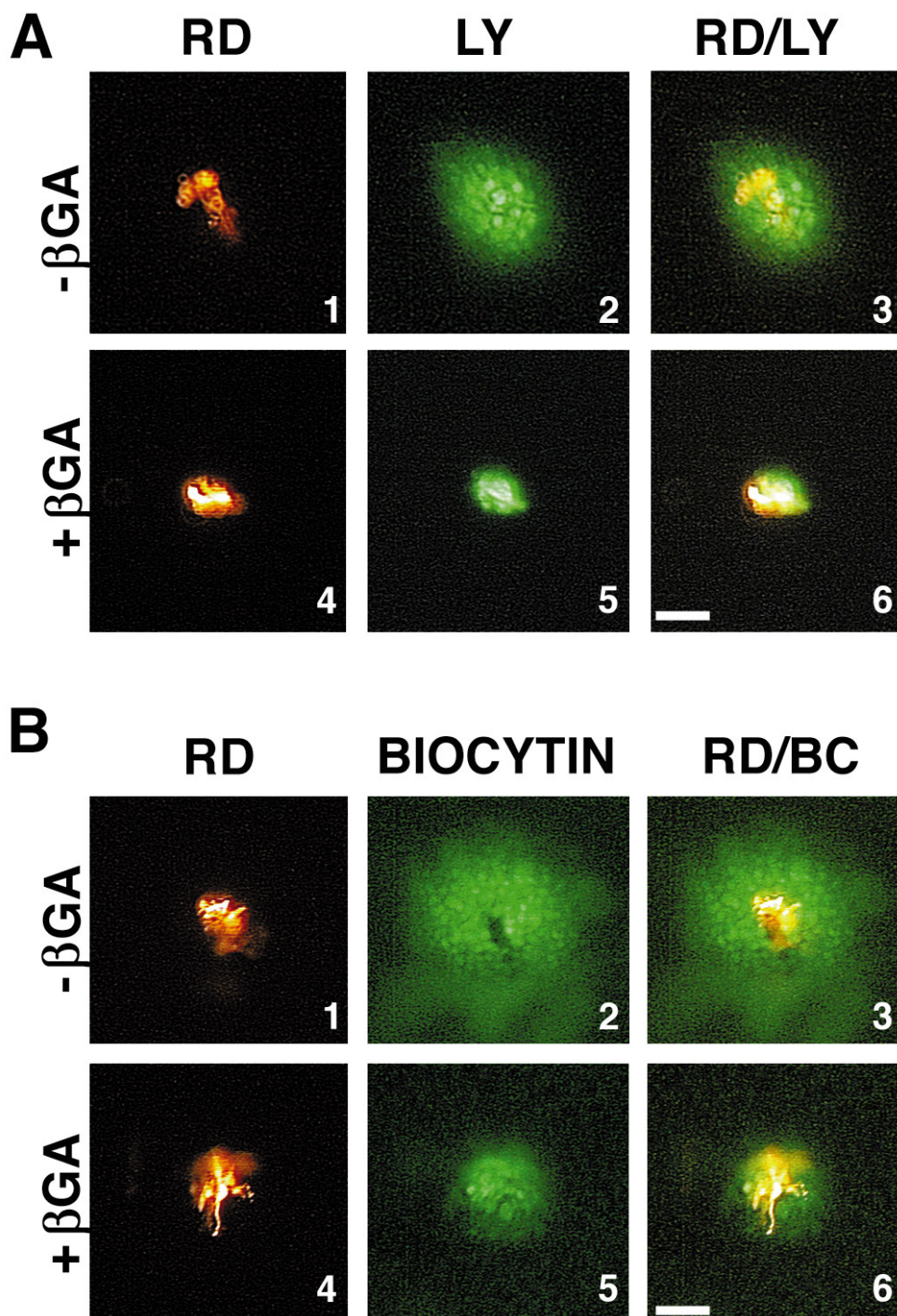


FIG. 10. β GA inhibits gap junction-mediated intercellular transfer of Lucifer yellow and biocytin in embryonic chick lens central epithelium explants. Lens epithelium explants cultured for 1 day in M199/15% fetal calf serum in either the absence (A1–A3; B1–B3) or presence (A4–A6; B4–B6) of 100 μ M 18 β -GA were scrape-loaded with rhodamine dextran mixed with either Lucifer yellow (A1–A6) or biocytin (B1–B6) as described under Materials and Methods. After 2 min, the cells were fixed and the dyes visualized by fluorescence microscopy. Gap junction-impermeant rhodamine dextran remained confined to the loaded cells immediately bordering the punctate wound (A1, A4; B1, B4). The extent to which Lucifer yellow (A2, A5) and the more sensitive gap junction tracer biocytin (B2, B5) are transferred to rhodamine dextran-negative cells is a measure of intercellular coupling. Superposition of the staining pattern of the two dyes clearly demonstrates the β GA-induced block of gap junction channels (compare A3 with A6; B3 with B6). Bar: 50 μ m.

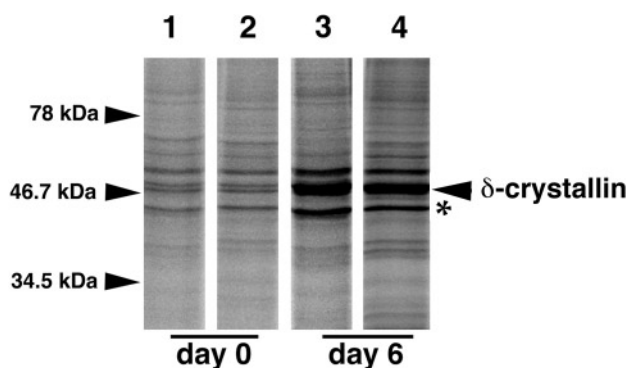


FIG. 11. β GA does not inhibit δ -crystallin expression in differentiating lens explants. Lens epithelium explants were metabolically labeled with 0.1 mCi/ml [35 S]methionine for 1 h in either the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 μ M 18 β -GA, either immediately following preparation (lanes 1 and 2) or after 6 days of culture in M199/15% fetal calf serum with (lane 4) or without (lane 3) 100 μ M 18 β -GA. The labeled cells were solubilized in SDS and equal amounts of total cell lysates were analyzed on SDS-PAGE. When normalized to actin (indicated by the asterisk) and quantitated with a PhosphorImager, synthesis of δ -crystallin in explants cultured in the continuous presence of β GA (lane 4) was $\approx 1.3\times$ of the untreated control (lane 3). Molecular weight markers are indicated on the left.

communication achieved by nontoxic doses of β GA is not absolute. This was expected, given that the β GA-related compound 18 α -carbenoxolone has been shown to reduce gap junction-mediated electrical coupling between tissue culture cells by only $\approx 75\%$ (Martin *et al.*, 1991; Goldberg *et al.*, 1996). Our experiments therefore do not rule out the possibility that a minimal level of gap junction-mediated communication is necessary for fiber differentiation, but do establish that the extensive up-regulation of gap junction formation and function that takes place in the bow region *in vivo* is not required for rapid and efficient epithelial-to-fiber differentiation in culture. Furthermore, our study does not address whether gap junctions play a role in stages of lens development that precede the time of lens harvest (E6 for explants and E10 for dissociated monolayer cultures) such as lens vesicle formation or primary fiber elongation, nor the function of gap junctions in very late developmental events (e.g., loss of nuclei) that are not recapitulated in our cultures.

A large number of studies have demonstrated that lens cells express receptors for a variety of growth factors and that their developmental fate is sensitive to the balance between proliferation-promoting and differentiating-promoting factors within the eye (Hyatt and Beebe, 1993; Lovicu *et al.*, 1995). Given the importance of extralenticular growth factors for lens development *in vivo*, it initially seemed surprising that our dissociated cell-derived cultures underwent epithelial-to-fiber differentiation in the absence of any exogenously added soluble or substrate-associated

growth factors. As discussed by Menko *et al.* (1984), the cells that are propagated in such cultures apparently originate from multiple areas of the lens, including the bow and annular pad regions. It therefore seems likely that at least some of the cells that developed into fibers in the absence of added growth factors were from these more peripheral zones which had already been "programmed" for fiber differentiation prior to the time (E9–10) of lens harvest. If so, then the possibility remained that the initial specification of epithelial cells for secondary fiber differentiation might have a requirement for high levels of gap junction-mediated intercellular communication. To address this issue, we used central epithelium explants comprised of the least differentiated population of cells within the lens. As expected from the literature (Piatigorsky, 1973), these explants survived in the absence of added growth factors but remained undifferentiated unless supplemented with 15% fetal calf serum, indicating that their developmental fate had not been predetermined *in vivo*. Gap junction-blocking levels of β GA did not, however, inhibit serum-mediated differentiation of these explants into MP28- and δ -crystallin-expressing fiber cells. Taken together, our results make it very unlikely that extensive gap junctional intercellular communication plays a role in either the induction or the execution of epithelial-to-secondary fiber differentiation.

Based on our findings, we predict that the concerted differentiation of adjacent cells that occurs in the bow region in the intact lens is coordinated by some mechanism other than increased gap junctional intercellular communication. One possibility consistent with the requirement for cell–cell contact would be paracrine signaling stimulated by an increase in the local concentration of a differentiation-inducing growth factor. The massive up-regulation of gap junction formation observed in the lens bow might instead be a consequence rather than a cause of fiber initiation and serve primarily to facilitate the intercellular transfer of metabolites involved in the burst of new protein synthesis that is essential for fiber formation. In addition, Mathias and co-workers (Mathias and Rae, 1985; Mathias *et al.*, 1997) have presented a compelling argument that the high level of gap junctional coupling at the equatorial region is required for the "microcirculation" of ions and fluids around and through the lens. A critical role for gap junctions in lens homeostasis is supported by the recent demonstration that elimination of Cx46 expression by targeted gene disruption results in the postnatal formation of nuclear cataracts in mice (Gong *et al.*, 1997).

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REFERENCES

- Allen, F., Tickle, C., and Warner, A. (1990). The role of gap junctions in patterning of the chick limb bud. *Development* **108**, 623–634.
- Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A., and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J Cell Biol.* **106**, 1141–1149.
- Arita, T., Lin, L. R., Susan, S. R., and Reddy, V. N. (1990). Enhancement of differentiation of human lens epithelium in tissue culture by changes in cell-substrate adhesion. *Invest. Ophthalmol. Visual Sci.* **31**, 2395–2404.
- Baldo, G. J., and Mathias, R. T. (1992). Spatial variations in membrane properties in the intact rat lens. *Biophys. J.* **63**, 518–529.
- Bennett, M. V. L., Barrio, L. C., Bargiello, T. A., Spray, D. C., Hertzberg, E., and Saez, J. C. (1991). Gap junctions: new tools, new answers, new questions. *Neuron* **6**, 305–320.
- Bevilacqua, A., Loch-Caruso, R., and Erickson, R. P. (1989). Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **86**, 5444–5448.
- Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1987). Connexin43: A protein from rat heart homologous to a gap junction protein from liver. *J Cell Biol.* **105**, 2621–2629.
- Berthoud, V. M., Cook, A. J., and Beyer, E. C. (1994). Characterization of the gap junction protein connexin56 in the chicken lens by immunofluorescence and immunoblotting. *Invest. Ophthalmol. Visual Sci.* **35**, 4109–4117.
- Bruzzone, R., White, T. W., and Goodenough, D. A. (1996). The cellular Internet: On-line with connexins. *BioEssays* **18**, 709–718.
- Chen, Y., Merzdorf, C., Paul, D. L., and Goodenough, D. A. (1997). COOH terminus of occludin is required for tight junction barrier function in early *Xenopus* embryos. *J. Cell Biol.* **138**, 981–989.
- Constantin B., Cronier, L., and Raymond, G. (1997). Transient involvement of gap junctional communication before fusion of newborn rat myoblasts. *CR Acad. Sci.- Ser. Iii* **320**, 35–40.
- Creighton, M. O., Mousa, G. Y., and Trevithick, J. R. (1976). Differentiation of rat lens epithelial cells in tissue culture. I. Effect of cell density, medium, and embryonic age of initial culture. *Differentiation* **6**, 155–167.
- Crow, J. M., Atkinson, M. M., and Johnson, R. G. (1994). Micromolar levels of intracellular calcium reduce gap junctional permeability in lens cultures. *Invest. Ophthalmol. Visual Sci.* **35**, 3332–3341.
- Davidson, J. S., Baumgarten, I. M., and Harley, E. H. (1986). Reversible inhibition of intercellular communication by glycyrrhetic acid. *Biochem. Biophys. Res. Commun.* **134**, 29–36.
- Desousa, P. A., Juneja, S. C., Caveney, S., Houghton, D. F., Davies, T. C., Reaume, A. G., Rossant, J., and Kidder, G. M. (1997). Normal development of preimplantation mouse embryos deficient in gap junctional coupling. *J. Cell Sci.* **110**, 1751–1758.
- Duband, J. L., Volberg, T., Sabanay, I., Thiery, J. P., and Geiger, B. (1988). Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development* **103**, 325–344.
- Edelman, G. M. (1988). Morphoregulatory molecules. *Biochemistry* **27**, 3533–3543.
- el-Fouly, M. H., Trosko, J. E., and Chang, C. C. (1987). Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* **168**, 422–430.
- Evans, C. W., Eastwood, S., Rains, J., Gruijters, W. T. M., Bullivant, S., and Kistler, J. (1993). Gap junction formation during development of the mouse lens. *Eur. J. Cell Biol.* **60**, 243–249.
- Frenzel, E. M., and Johnson, R. G. (1996). Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. *Dev. Biol.* **179**, 1–16.
- Goldberg, G. S., Moreno, A. P., Bechberger, J. F., Hearn, S. S., Shivers, R. R., MacPhee, D. J., Zhang, Y. C., and Naus, C. C. (1996). Evidence that disruption of connexon particle arrangements in gap junction plaques is associated with inhibition of gap junctional communication by a glycyrrhetic acid derivative. *Exp. Cell Res.* **222**, 48–53.
- Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N. M., Horwitz, J., and Gilula, N. B. (1997). Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. *Cell* **91**, 833–843.
- Goodenough, D. A. (1992). The crystalline lens. A system networked by gap junctional intercellular communication. *Semin. Cell Biol.* **3**, 49–58.
- Goodenough, D. A., Dick, J. S. B., II, and Lyons, J. E. (1980). Lens metabolic cooperation: a study of mouse lens transport and permeability visualized with freeze-substitution autoradiography and electron microscopy. *J. Cell Biol.* **86**, 576–589.
- Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.* **65**, 475–502.
- Guan, X., Wilson, S., Schlender, K. K., and Ruch, R. J. (1996). Gap-junction disassembly and connexin 43 dephosphorylation induced by 18 β -glycyrrhetic acid. *Mol. Carcinogen.* **16**, 157–164.
- Gumbiner, B., Stevenson, B., and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* **107**, 1575–1587.
- Guthrie, S. C., and Gilula, N. B. (1989). Gap junctional communication and development. *Trends Neurosci.* **12**, 12–16.
- Hirano, S., Nose, A., Hatta, K., Kawakami, A., and Takeichi, M. (1987). Calcium-dependent cell-cell adhesion molecules (cadherins): Subclass specificities and possible involvement of actin bundles. *J. Cell Biol.* **105**, 2501–2510.
- Hyatt, G. A., and Beebe, D. C. (1993). Regulation of lens cell growth and polarity by an embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation. *Development* **117**, 701–709.
- Jiang, J. X., White, T. W., Goodenough, D. A., and Paul, D. L. (1994). Molecular cloning and functional characterization of chick lens fiber connexin 45.6 *Mol Biol Cell* **5**, 363–373.
- Jiang, J. X., White, T. W., and Goodenough, D. A. (1995). Changes in connexin expression and distribution during chick lens development. *Dev. Biol.* **168**, 649–661.

- Kreft, B., Berndorff, D., Bottinger, A., Finnemann, S., Wedlich, D., Hortsch, M., Tauber, R., and Gessner, R. (1997) LI-cadherin-mediated cell-cell adhesion does not require cytoplasmic interactions. *J. Cell Biol.* **136**, 1109–1121.
- Lee, S., Gilula, N. B., and Warner, A. E. (1987). Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell* **51**, 851–860.
- Lo, C. W. (1996) The role of gap junction membrane channels in development. *J. Bioenergetics Biomembr.* **28**, 379–385.
- Lovicu, F. J., Chamberlain, C. G., and McAvoy, J. W. (1995). Differential effects of aqueous and vitreous on fiber differentiation and extracellular matrix accumulation in lens epithelial explants. *Invest. Ophthalmol. Visual Sci.* **36**, 1459–1469.
- Martin, W., Zempel, G., Hulser, D., and Willecke, K. (1991). Growth inhibition of oncogene-transformed rat fibroblasts by cocultured normal cells: Relevance of metabolic cooperation mediated by gap junctions. *Cancer Res.* **51**, 5348–5351.
- Mathias, R. T., and Rae, J. L. (1985). Transport properties of the lens. *Am. J. Physiol.* **249**, C181–C190.
- Mathias, R. T., Rae, J. L., Baldo, G. J. (1997). Physiological properties of the normal lens. *Physiol. Rev.* **77**, 21–50.
- Mege, R. M., Goudou, D., Giaume, C., Nicolet, M., and Rieger, F. (1994). Is intercellular communication via gap junctions required for myoblast fusion? *Cell Adhesion Commun.* **2**, 329–343.
- Menko, A. S., and Boettiger, D. (1988). Inhibition of chicken embryo lens differentiation and lens junction formation in culture by pp60v-src. *Mol. Cell. Biol.* **8**, 1414–1420.
- Menko, A. S., Klukas, K. A., and Johnson, R. G. (1984). Chicken embryo lens cultures mimic differentiation in the lens. *Dev. Biol.* **103**, 129–141.
- Menko, A. S., Klukas, K. A., Liu, T. F., Quade, B., Sas, D. F., Preus, D. M., and Johnson, R. G. (1987). Junctions between lens cells in differentiating cultures: Structure, formation, intercellular permeability, and junctional protein expression. *Dev. Biol.* **123**, 307–320.
- Meyer, R. A., Laird, D. W., Revel, J. P., and Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell Biol.* **119**, 179–189.
- Miller, T. M., and Goodenough, D. A. (1986). Evidence for two physiologically distinct gap junctions expressed by the chick lens epithelial cell. *J. Cell Biol.* **102**, 194–199.
- Musil, L. S., Beyer, E. C., and Goodenough, D. A. (1990a). Expression of the gap junction protein connexin43 in embryonic chick lens: Molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J. Membr. Biol.* **116**, 163–175.
- Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990b). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* **111**, 2077–2088.
- Musil, L. S., and Goodenough, D. A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* **115**, 1357–1374.
- Paul, D. L., Ebihara, L., Takemoto, L. J., Swenson, K. I., and Goodenough, D. A. (1991). Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. *J. Cell Biol.* **115**, 1077–1089.
- Paul, D. L., Yu, K., Bruzzone, R., Gimlich, R. L., and Goodenough, D. A. (1995). Expression of a dominant negative inhibitor of intercellular communication in the early *Xenopus* embryo causes delamination and extrusion of cells. *Development* **121**, 371–381.
- Philpott, G. W., and Coulombre, A. J. (1968). Cytodifferentiation of precultured embryonic chick lens epithelial cells *in vitro* and *in vivo*. *Exp. Cell Res.* **52**, 140–146.
- Piatigorsky, J. (1973). Insulin initiation of lens fiber differentiation in culture: elongation of embryonic lens epithelial cells. *Dev. Biol.* **30**, 214–216.
- Piatigorsky, J. (1981). Lens differentiation in vertebrates. A review of cellular and molecular features. *Differentiation* **19**, 134–153.
- Piatigorsky, J., Webster, H. D., and Craig, S. P. (1972). Protein synthesis and ultrastructure during the formation of embryonic chick lens fibers *in vivo* and *in vitro*. *Dev. Biol.* **27**, 176–189.
- Piatigorsky, J., Rothschild, S. S., and Milstone, L. M. (1973). Differentiation of lens fibers in explanted embryonic chick lens epithelia. *Dev. Biol.* **34**, 334–345.
- Proulx, A., Merrifield, P. A., and Naus, C. C. (1997a). Blocking gap junctional intercellular communication in myoblasts inhibits myogenin and MRF4 expression. *Dev. Genet.* **20**, 133–144.
- Proulx, A., Lin Z. X., and Naus, C. C. (1997b). Transfection of rhabdomyosarcoma cells with connexin43 induces myogenic differentiation. *Cell Growth Differ.* **8**, 533–540.
- Reaume, A. G., De Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M., and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**, 1831–1834.
- Richardson, N. A., McAvoy, J. W., and Chamberlain, C. G. (1992). Age of rats affects response of lens epithelial explants to fibroblast growth factor. *Exp. Eye Res.* **55**, 649–656.
- Rup, D. M., Veenstra, R. D., Wang, H.-Z., Brink, P. R., and Beyer, E. C. (1993). Chick connexin-56, a novel lens gap junction protein. *J. Biol. Chem.* **268**, 706–712.
- Sas, D. F., Sas, M. J., Johnson, K. R., Menko, S., and Johnson, R. G. (1985). Junctions between lens fiber cells are labeled with a monoclonal antibody shown to be specific for MP26. *J. Cell Biol.* **100**, 216–225.
- Simon A. M., Goodenough, D. A., Li, E., and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. *Nature* **385**, 525–529.
- Statuto, M., Audebet, C., Tonoli, H., Selmi-Ruby, S., Rousset, B., and Munari-Silem, Y. (1997). Restoration of cell-to-cell communication in thyroid cell lines by transfection with and stable expression of the connexin-32 gene. *J. Biol. Chem.* **272**, 24710–24716.
- Stein, L. S., Boonstra, J., Burghardt, R. C. (1992). Reduced cell-cell communication between mitotic and nonmitotic coupled cells. *Exp. Cell Res.* **198**, 1–7.
- TenBroek, E. M., Johnson, R., and Louis, C. F. (1994). Cell-to-cell communication in a differentiating ovine lens culture system. *Invest. Ophthalmol. Visual Sci.* **35**, 215–228.
- Teranishi, T., and Negishi, K. (1994). Double-staining of horizontal and amacrine cells by intracellular injection with lucifer yellow and biocytin in carp retina. *Neuroscience* **59**, 217–226.
- Umino, O., Maehara, M., Hidaka, S., Kita, S., and Hashimoto, Y. (1994). The network properties of bipolar-bipolar cell coupling in the retina of teleost fishes. *Visual Neurosci.* **11**, 533–548.
- Volk, T., and Geiger, B. (1986). A-CAM: A 135-kD receptor of intercellular adherens junctions. II. Antibody-mediated modulation of junction formation. *J. Cell Biol.* **103**, 1451–1464.
- Vozzi C., Ullrich, S., Charollais, A., Philippe, J., Orci, L., and Meda, P. (1995). Adequate connexin-mediated coupling is required for proper insulin production. *J. Cell Biol.* **131**, 1561–1572.

- Warner, A. E., Guthrie, S. C., and Gilula, N. B. (1984). Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* **311**, 127–131.
- Warner, A. E. (1992). Gap junctions in development—A perspective. *Semin. Cell Biol.* **3**, 81–91.
- Watanabe, M., Kobayashi, H., Rutishauser, U., Katar, M., Alcala, J., and Maisel, H. (1989). NCAM in the differentiation of embryonic lens tissue. *Dev. Biol.* **135**, 414–423.
- White, T. W., Bruzzone, R., Goodenough, D. A., and Paul, D. L. (1992). Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol. Biol. Cell* **3**, 711–720.
- Wong, V., and Gumbiner, B. M. (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J. Cell Biol.* **136**, 399–409.
- Wride, M. A. (1996). Cellular and molecular features of lens differentiation: A review of recent advances. *Differentiation* **61**, 77–93.
- Yancey, S. B., Koh, K., Chung, J., and Revel, J.-P. (1988). Expression of the gene for the main intrinsic polypeptide (MIP): Separate spatial distributions of MIP and β -crystallin gene transcripts in rat lens development. *J. Cell Biol.* **106**, 705–714.
- Zampighi, G., Simon, S. A., and Hall, J. E. (1992) The specialized junctions of the lens. *Int. Rev. Cytol.* **136**, 185–225.

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